

Maternal deprivation promotes hippocampal neuronal apoptosis via ERK1/2 signaling

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Methods
 - 3.1. Animals
 - 3.2. Maternal deprivation regimen
 - 3.3. Behavioral tests
 - 3.4. Flow cytometry
 - 3.5. TUNEL staining
 - 3.6. Nissl staining
 - 3.7. Immunohistochemistry
 - 3.8. Real-time PCR
 - 3.9. Western blotting
 - 3.10. Statistical analysis
4. Results
 - 4.1. Maternal-deprived rats show depressive-like behaviors
 - 4.2. Maternal deprivation promotes apoptosis in hippocampal neurons
 - 4.3. Maternal deprivation inhibits mRNA expression of ERK1/2 signaling components
 - 4.4. Maternal deprivation inhibits protein expression of ERK1/2 signaling components
5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

Neonatal maternal deprivation (MD) leads to depressive-like behaviors by promoting hippocampal neuronal apoptosis, but whether and how ERK1/2 signaling participates in MD-induced hippocampal neuronal apoptosis remains unclear. We therefore explored the role of ERK1/2 signaling pathway in neonatal MD-induced depression. Neonatal rats were divided into control group, PD group given intraperitoneal injections of MAPKK inhibitor (PD98059), MD group, and MD+PD group. The neonatal MD model was confirmed by behavioral tests. Compared with controls, MD group showed increased hippocampal neuronal apoptosis, higher mRNA expressions of Bax, caspase-3 and caspase-9mRNAs, and lower expression of Bcl-2 and BDNF. Similarly, compared with controls, MD group showed higher protein expression and wider distribution of Bax, caspase-3, caspase-9 and Cytochrome C but

lower protein expression of Bcl-2, p-CREB, BDNF and p-ERK1/2. The changes in the MD group were reversed in the MD+PD group, except that p-ERK1/2 expression was decreased. In conclusion, MD-induced depression is associated with hippocampal neuronal apoptosis, which may be mediated by the ERK1/2 signaling pathway. These findings may provide novel avenues for depression therapy.

2. INTRODUCTION

Worldwide, major depressive disorder imposes a major health burden, as approximately 300 million people were affected in 2010 (1). It is predicted to become one of the three leading causes of the disease burden by 2030 (2). The occurrence of depressive disorders is associated with early maternal and peer separations (3). However, it remains unclear

how stress in early life promotes depressive disorders in adults. Studies in animals have found that neonatal maternal deprivation (MD) may confer a long-term and negative effect on neuroimmunoendocrine interactions, leading to depressive-like behavior (4,5). Furthermore, MD leads to changes in brain structure and to neuronal apoptosis, predominantly in the hippocampal formation (6-8). However, the precise mechanisms have not been clarified.

It has been reported that the activity of the extracellular signal-regulated kinase (ERK) signaling pathway in reducing apoptosis is decreased in patients with depression (9). ERK1 and ERK2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) family. On the one hand, ERK1/2 can regulate the expression of brain-derived neurotrophic factor (BDNF) and cAMP responsive element-binding protein (CREB) (10,11). ERK1/2 phosphorylation could also lead to p-CREB, resulting in behavior changes in cells (12). Specifically, phosphorylated CREB (p-CREB) can activate BDNF, which can promote neuronal growth in the hippocampus (13,14). BDNF is crucial for neurogenesis and enhances neuronal survival (15). Moreover, both BDNF and CREB are related to depression (16-18). On the other hand, the activation and phosphorylation of ERK1/2 (p-ERK1/2) can regulate the activity of caspase-3 (19), which is related to apoptosis associated with mitochondrial dysfunction. That process is accompanied by the activation of Cytochrome C, B-cell lymphoma-2 (Bcl-2), Bax and caspase-9.

However, it is unclear whether and how ERK1/2 signaling participates in MD-induced hippocampal neuronal apoptosis. Therefore, we conducted the present study with a rat model to explore the role of the ERK1/2 signaling pathway in neonatal MD-induced depression.

3. METHODS

3.1. Animals

Adult Sprague-Dawley rats (40 females and 40 males) weighing 200-250 g were purchased from Charles River (Beijing, China). One female and one male rat were housed per cage. When the female rat became pregnant, it was moved to a separate cage. The neonatal rats were divided into four groups as follows: control, PD with intraperitoneal injections of a MAPKK inhibitor (PD98059, 10 mg/kg), MD, and MD+PD. Each group contained 20 neonatal rats.

3.2. Maternal deprivation regimen

The maternal deprivation paradigm was developed as previously reported (20). From the first day to the 14th day, neonatal rats were removed

from their mothers' home cage, moved to a separate standard polycarbonate box, all together, and subjected to maternal deprivation for 6 hours per day, from 9 am to 3 pm. The MD box temperature was monitored and maintained at 25°C. After the 6 hours, the neonatal rats were returned to their mothers.

3.3. Behavioral tests

A sucrose preference test to assess the degree of anhedonia in neonatal rats was performed as previously described (21). On the first day, rats were housed individually and given free access to two bottles of sucrose solution (1%, w/v) for 24 hours. On the second day, one bottle of sucrose solution was replaced with water for 24 hours. On the third day, rats were deprived of water and food for 23 h, and during the last 1 h, rats were given free access to two pre-weighed bottles of solution. The positions of the two bottles were switched compared to the second day. The amounts (in grams) of sucrose and water consumed in both bottles were recorded. The sucrose preference rate was calculated as follows: sucrose preference rate = sucrose consumption (g)/(water consumption (g)+sucrose consumption (g)).

A forced swim test was used to measure behavioral despair according to a previously established protocol (22). During testing, rats were placed individually in a transparent cylinder containing water (25°C) at a depth of 30 cm. After swimming for 15 min on the first day, rats were dried with towels and placed back in their home cages. At the end of each trial, to reduce interference between animals, the cylinder was emptied and then refilled with water. Twenty-four hours after the first trial, rats were placed in the swimming apparatus again for a 5-min test trial. The immobility time (the amount of time a rat spent keeping its head above water with only slight movements) was calculated.

An open-field test was used to assess anxiety-induced locomotor activity and exploratory behavior (23). Neonatal rat movements and behaviors were recorded during 5-minute sessions in an open field (100×100×40 cm). Rats were placed individually into the center of the arena, at which time a video camera suspended over the center of the arena began recording. The experimenter then immediately left the room, and the animal was left in the arena for 5 min. After 5 min, the animal was removed from the arena and placed back into its home cage. The arena was then cleaned with a 70% ethanol solution. The floor was divided into a grid of 25 squares of 20×20 cm each. The 9 squares in the center were considered the "center zone", and the other 16 squares were considered the "peripheral zone". The walking distance, number of defecations, number of times rearing, and percentage of time spent in the center zone were recorded.

3.4. Flow cytometry

Neurons from the hippocampus were collected and subjected to treatment with an AnnexinV-FITC/PI Apoptosis Detection Kit (ComWin Biotech, Beijing, China) according to the manufacturer's instructions. Cellular apoptosis was detected with flow cytometry. Cells positive for ANNEXIN V alone were recognized as early apoptotic, cells positive for PI were recognized as necrotic, and cells positive for both ANNEXIN V and PI were recognized as late apoptotic.

3.5. TUNEL stainings

The formalin-fixed and paraffin-embedded hippocampal tissues were cut into 3- μ m-thick sections. Sections were fixed with acetone for 10 min and quenched with 2% H₂O₂ for 5 min. TUNEL staining was detected using a TUNEL kit (keyGEN BioTECH, Nanjing, China) according to the manufacturer's instructions. Images were taken with a microscope equipped with DP801 software (Olympus, Japan). Each tissue sample was cut into 5 slides. The neuronal density in the CA1 to CA4 regions was quantified in three fields per region. The cases were coded, and the measurements were made in a blinded fashion by two pathologists blinded to the group of a given specimen. Disagreements were settled by consensus or adjudicated by a third reviewer.

3.6. NISSL staining

Hippocampal tissues were fixed with formalin, embedded with paraffin, and cut in 3- μ m-thick sections. The sections were stained with 0.1% cresyl violet for 15-30 min at room temperature and then assessed for neuronal death by using a light microscope (Olympus CX31R BSF, Japan).

3.7. Immunohistochemistry

The formalin-fixed and paraffin-embedded hippocampal tissues were cut in 3- μ m-thick sections. To detect immunoreactivity with the peroxidase method, endogenous peroxides were quenched with 0.3% H₂O₂ for 10 minutes. Sections were blocked with normal goat serum or normal rabbit serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The slides were then incubated overnight at 4°C with rabbit anti-rat p-ERK1/2 antibody (1:1000; Cell Signaling Technology, MA, US), Bcl-2 antibody (1:1500; Cell Signaling Technology, MA, US), Bax antibody (1:1000; Cell Signaling Technology, MA, US), caspase-3 antibody (1:1000; Cell Signaling Technology, MA, US), or caspase-9 antibody (1:1000; Cell Signaling Technology, MA, US) and with mouse anti-rat BDNF antibody (1:1000; BOSTER, Wuhan, China), p-CREB antibody (1:1000; BOSTER, Wuhan, China), or Cytochrome C antibody (1:2000; BOSTER, Wuhan,

China). The resulting slides were incubated at room temperature with biotinylated secondary antibody and streptavidin, for 20 min each. 3,3'-Diaminobenzidine tetrahydrochloride was applied as a chromogen for 5-10 min. Sections were counterstained in hematoxylin for 1 min.

3.8. Real-time PCR

RNA from hippocampal tissues was isolated by using RNAiso Reagent (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The cDNA was synthesized with a SYBR ExScript TMRT-PCR Kit (Sangon Biotech, Shanghai, China). Quantitative real-time polymerase chain reaction (PCR) was performed with an ABI7300 instrument (Applied Biosystems, US) and SYBR Premix Ex Taq (TAKARA, Japan) in accordance with the manufacturer's protocol. Each set of experiments was repeated three times. The 25- μ l PCR amplifications (with 12.5 μ l SYBR green, 0.5 μ l (10 μ mol/L) forward and 0.5 μ l (10 μ mol/L) reverse primers, and 2 μ l cDNA) underwent 10 s at 95°C, 5 s at 95°C, then 40 cycles of 31 s at 60°C. Primers were as follows: Bax F, 5'-TTG CTA CAG GGT TTC ATC CA-3'; Bax R, 5'-TGT TGT TGT CCA GTT CAT CG-3'; Bcl-2 F, 5'-GAG CGT CAA CAG GGA GAT GT-3'; Bcl-2 R, 5'-CAG CCA GGA GAA ATC AAA CAG-3'; BDNF F, 5'-ACT GCC ACT GAA ATG CGA CT-3'; BDNF R, 5'-GCT TCC GAG CCT TCC TTT AG-3'; caspase 3 F, 5'-AGC TGG ACT GCG GTA TTG AG-3'; caspase 3 R, 5'-GGG TGC GGT AGA GTA AGC AT-3'; caspase 9 F, 5'-GTG AAG AAC GAC CTG ACT GC-3'; and caspase 9 R, 5'-AGA GGA TGA CCA CCA CGA AG-3'. The threshold cycle number (Ct) was determined for all PCR amplifications, and in most cases, the threshold was manually adjusted to lie within the exponential phase by using Sequence Detection software version 1.2.3. (Applied Biosystems, US).

3.9. Western blotting

Hippocampal tissues were lysed in 0.5 ml lysis buffer, and protein was then extracted using a Protein Extraction Kit (Beyotime, Shanghai, China). Protein was separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to PVDF (Solarbio, Beijing, China), followed by blocking in 1×TBS-Tween 20 with 5% skim milk. The membranes were incubated overnight at 4°C with rabbit anti-rat p-ERK1/2 antibody (1:1000; Cell Signaling Technology, MA, US), Bcl-2 antibody (1:1500; Cell Signaling Technology, MA, US), Bax antibody (1:1000; Cell Signaling Technology, MA, US), caspase-3 antibody (1:1000; Cell Signaling Technology, MA, US), caspase-9 antibody (1:1000; Cell Signaling Technology, MA, US), or ERK1/2 antibody (1:1000; Cell Signaling Technology, MA, US) and with mouse anti-rat BDNF antibody (1:1000; BOSTER, Wuhan, China), p-CREB antibody (1:1000; BOSTER, Wuhan, China),

Table 1. Behavioral tests of neonatal rats

Behavioral test	Control group (n=20)	PD group (n=20)	MD group (n=20)	MD+PD group (n=20)
Sucrose preference test				
Water consumption (g)	6.10±0.99	7.60±1.84	12.80±2.97*	10.60±2.50*
Sucrose consumption (g)	11.70±2.58	12.70±1.49	11.70±1.89	12.20±1.75
Sucrose preference rate (%)	59.82±4.35	55.29±9.13	50.66±4.01*	55.78±4.02*#
Forced swim test				
Immobility time (s)	65.45±20.57	70.55±21.41	148.15±41.76 *	119.30±33.65 *#
Open-field test				
Crawling distance (cm)	1210±353.96	1234±334.04	750±342.34*	944±435.36 #
Proportion of time spent in the center zone (%)	15.10±11.17	15.70±9.24	19.95±10.62	16.30±9.18
Number of defecations	4.45±2.87	3.50±3.07	2.55±2.19	3.35±2.72
Number of times rearing	13.95±7.61	13.55±4.91	9.70±4.62	12.40±6.49

MD: maternal deprivation; PD: PD98059, *: P <0.05 compared with control group, #: P <0.05 compared with MD group.

Cytochrome C antibody (1:2000; BOSTER, Wuhan, China), CREB antibody (1:1000; BOSTER, Wuhan, China) or GAPDH antibody (1:2000) (Santa Cruz Biotechnology, CA, USA), followed by incubation for 2 h at room temperature with goat anti-rabbit or goat anti-mouse IgG HRP-conjugated antibody (1:10,000; ComWin Biotech, Beijing, China).

3.1.0. Statistical analysis

Group data are expressed as the mean±standard deviation (SD). Significant differences were evaluated using one-way analysis of variance (ANOVA) followed by the least significant difference test or the Games-Howell test. P values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS 19.0. (IBM SPSS Inc., Chicago, IL, USA).

4. RESULTS

4.1. Maternal-deprived rats show depressive-like behaviors

Table 1 illustrates the results of the behavioral tests. The MD group had a lower sucrose preference rate (50.66±4.01%) and a longer swim immobility time (148.15±41.76 s) than controls (sucrose preference rate: 59.82±4.35%; immobility time: 65.45±20.57 s), which could be ameliorated by PD98059 (sucrose preference rate: 55.78±4.02%; immobility time: 119.30±33.65 s). In the open-field test, the crawling distance of MD rats (750±342.34 cm) was decreased compared with controls (1210±353.96 cm). PD98059 increased the crawling distance of MD rats (944±435.36 cm), but this effect was not significant. The percentages of time spent in the center zone and the numbers of defecations and times rearing were similar among all groups.

4.2. Maternal deprivation promotes apoptosis in hippocampal neurons

The percentage of apoptotic neurons in the MD group (14.70±0.52%) was significantly higher than in controls (8.90±0.11%), whereas PD98059 inhibited neuronal apoptosis in MD rats (9.80±0.23%). The percentage of apoptotic neurons in the PD group was 9.27±0.27%. Both TUNEL staining and Nissl staining showed results consistent with those from flow cytometry (Figure 1).

4.3. Maternal deprivation inhibits mrna expression of erk1/2 signaling components

The mRNA expression levels of Bcl-2 (0.41±0.04) and BDNF (0.50±0.05) in the MD group were lower than in the controls (Bcl-2: 1.00±0.08; BDNF: 1.01±0.11), and those of Bax (1.41±0.19), caspase-3 (2.16±0.59) and caspase-9 (1.76±0.41) were higher than in the controls (Bax: 1.00±0.05; caspase-3: 1.00±0.06; caspase-9: 1.00±0.05). However, these effects of MD were reversed by PD98059 in the MD+PD group (Bcl-2: 0.59±0.04; Bax: 1.18±0.05; caspase-3: 1.47±0.10; caspase-9: 1.25±0.08; BDNF: 0.73±0.06). The expression levels in the PD group (Bcl-2: 0.94±0.16; Bax: 1.03±0.08; caspase-3: 1.11±0.11; caspase-9: 0.93±0.07; BDNF: 0.95±0.11) were not significantly different from controls (Figure 2).

4.4. Maternal deprivation inhibits protein expression of erk1/2 signaling components

Immunohistochemistry shows that in the hippocampal neurons, there were fewer Bcl-2+ cells (4.49±0.69), BDNF+ cells (3.02±0.41), p-CREB+ cells (1.98±0.34), and p-ERK1/2+ cells (3.68±0.45)

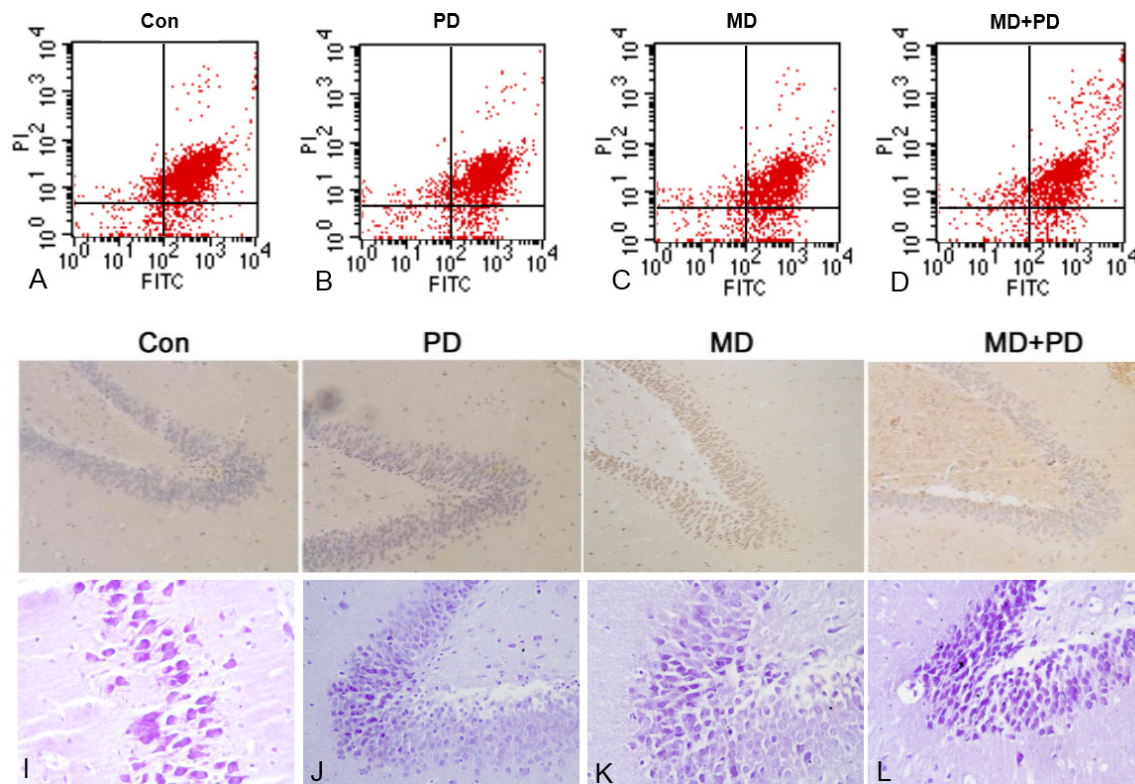


Figure 1. Neuronal apoptosis. (A-D) Flow cytometry. (E-H) TUNEL staining ($\times 200$). (I-L) Nissl staining ($\times 200$). Con: control; MD: maternal deprivation; PD: PD98059.

and more Bax⁺ cells ($13.2.7 \pm 0.8.7$), caspase-3⁺ cells ($7.9.0 \pm 1.3.9$), caspase-9⁺ cells ($8.4.7 \pm 1.5.7$), and Cytochrome C⁺ cells ($9.5.8 \pm 1.1.1$) in the MD group than in the control group (Bcl-2⁺ cells: $8.3.6 \pm 1.1.8$; BDNF⁺ cells: $6.2.4 \pm 1.6.4$; p-CREB⁺ cells: $4.3.7 \pm 0.6.9$; p-ERK1/2⁺ cells: $6.7.0 \pm 0.9.3$; Bax⁺ cells: $8.7.1 \pm 0.9.6$; caspase-3⁺ cells: $3.7.1 \pm 1.1.3$; caspase-9⁺ cells: $4.4.5 \pm 0.7.2$; Cytochrome C⁺ cells: $4.3.0 \pm 0.8.1$). However, these changes were reversed by PD98059 in the MD+PD group (Bcl-2⁺ cells: $6.8.6 \pm 0.5.6$; BDNF⁺ cells: $4.5.6 \pm 0.5.4$; p-CREB⁺ cells: $2.9.3 \pm 0.3.3$; Bax⁺ cells: $10.5.4 \pm 0.9.9$; caspase-3⁺ cells: $6.4.2 \pm 0.7.6$; caspase-9⁺ cells: $6.1.6 \pm 0.4.6$; Cytochrome C⁺ cells: $6.3.8 \pm 1.4.7$), with the exception of p-ERK1/2⁺ cells ($1.1.5 \pm 0.3.9$). In the PD group, expression of most of these proteins was comparable to that in the controls (Bcl-2⁺ cells: $8.5.5 \pm 0.8.6$; BDNF⁺ cells: $6.2.8 \pm 0.9.2$; p-CREB⁺ cells: $4.2.8 \pm 0.9.6$; Bax⁺ cells: $8.5.7 \pm 0.7.1$; caspase-3⁺ cells: $4.1.8 \pm 0.8.2$; caspase-9⁺ cells: $4.2.2 \pm 0.5.5$; Cytochrome C⁺ cells: $4.5.8 \pm 0.6.4$). However, there were fewer p-ERK1/2⁺ cells ($1.6.2 \pm 0.3.3$) in the PD group than in the control group (Figure 3,4).

Western blotting shows that in the hippocampus, the protein expression levels of Bcl-2, BDNF, p-CREB, and p-ERK1/2 were lower and that

those of Bax, caspase-3, caspase-9, and Cytochrome C were higher in the MD group than in the control group, effects that were reversed by PD98059 in the MD+PD group, with the exception of the decrease in p-ERK1/2. Expression levels of nearly all of these proteins were not significantly different between the PD group and the controls. However, the PD group showed lower p-ERK1/2 expression and higher Bax expression than in controls (Figure 5).

5. DISCUSSION

In this study, we found that MD promoted neuronal apoptosis in the hippocampus. MD also reduced the expression of components of the p-ERK1/2-BDNF-CREB pathway but increased the expression of Bax, caspase-3, caspase-9, and Cytochrome C. These effects of MD could be reversed by a MAPKK inhibitor. These findings suggest that MD may promote neuronal apoptosis in the hippocampus through the ERK1/2 signaling pathway.

The behavioral tests show that compared to the control group, the MD group had a lower sucrose preference rate and a longer swim immobility time but worse performance in an open-field test, and these effects were reversed by a MAPKK inhibitor.

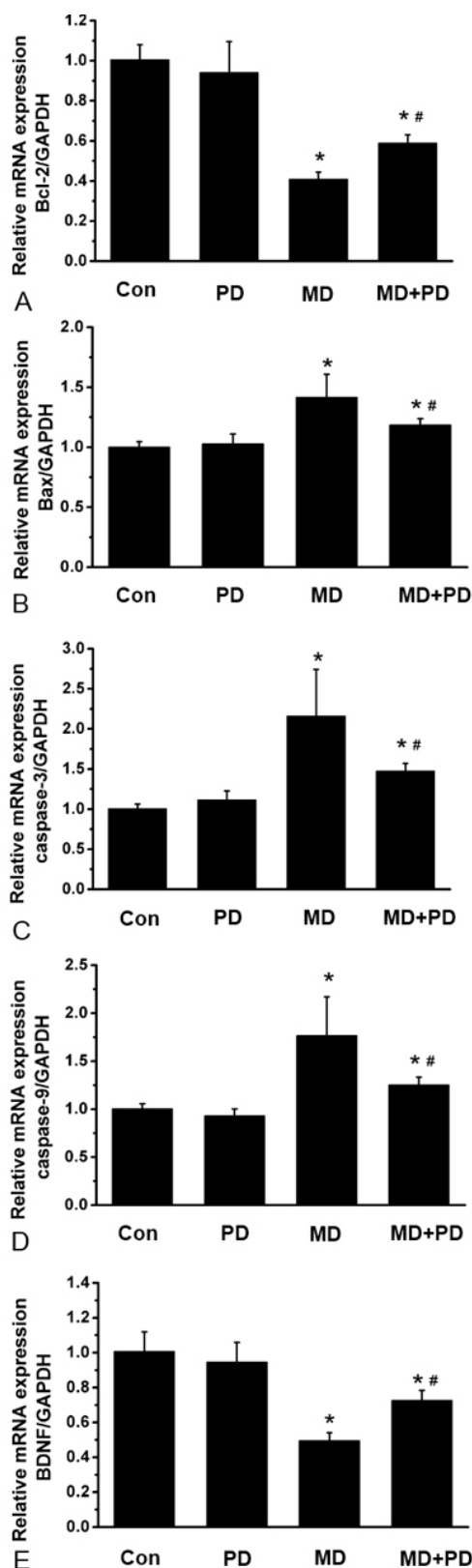


Figure 2. mRNA expression levels of Bcl-2, Bax, caspase-3, caspase-9, and BDNF in the hippocampus. Con: control; MD: maternal deprivation; PD: PD98059. *: $P < 0.05$ compared with control group. #: $P < 0.05$ compared with MD group.

The behavioral tests confirm the establishment of an MD-induced depression model. Moreover, the effect of a MAPKK inhibitor on MD rats suggests that ERK signaling may participate in MD-induced depression.

The results from flow cytometry, TUNEL staining and Nissl staining confirm previous reports that MD could induce neuronal apoptosis in the hippocampus (24,25). Since the MD rats showed depressive-like behaviors compared with controls, the increased neuronal apoptosis in the hippocampus may be associated with the depressive phenotype of MD rats. This is consistent with previous studies (26,27). Interestingly, our study shows that a MAPKK inhibitor reduced hippocampal neuronal apoptosis. This finding suggests that ERK1/2 signaling may cause MD rats to show depressive behaviors by inducing hippocampal neuronal apoptosis.

We further explored the possible mechanisms by which ERK1/2 signaling induces hippocampal neuronal apoptosis. On one hand, we found that both mRNA and protein expression levels of BDNF as well as p-CREB protein expression in the hippocampus were decreased in the MD group and that these effects were reversed by a MAPKK inhibitor. Considering the role of p-CREB and BDNF in neuronal survival and depression (13-15), these findings suggest that ERK1/2 signaling may promote hippocampal neuronal apoptosis by down-regulating the p-CREB/BDNF pathway.

On the other hand, the MD group showed decreased hippocampal expression of Bcl-2 but increased hippocampal expression of Bax, caspase-3, caspase-9 and Cytochrome C compared with controls. These effects of MD were also reversed by a MAPKK inhibitor. Thus, ERK1/2 signaling could promote mitochondrial dysfunction-related apoptosis. This is consistent with previous studies showing that ERK1/2 signaling activation reduces neuronal apoptosis in the hippocampus of embryonic and neonatal rats (128,29). Interestingly, the p-ERK1/2 protein expression was decreased in both the MD and PD groups. This suggests that p-ERK1/2 may play a role in inhibiting neuronal apoptosis during MD.

In conclusion, our study demonstrates that MD-induced depression is associated with hippocampal neuronal apoptosis, which may be mediated by ERK1/2 signaling through the CREB-BDNF pathway and by mitochondrial dysfunction. These findings may provide novel avenues for depression therapy.

6. ACKNOWLEDGEMENTS

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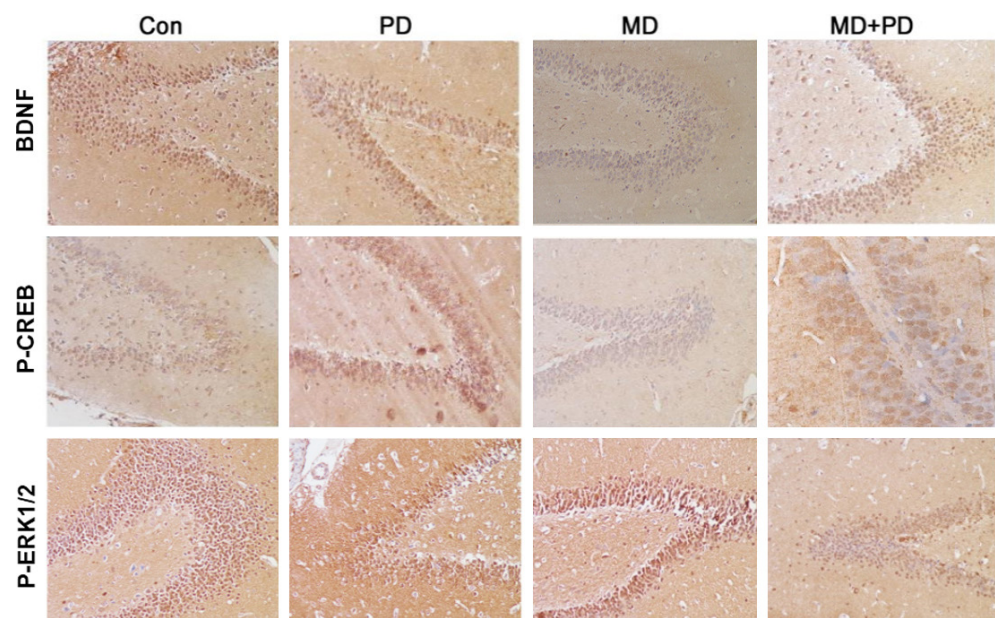


Figure 3. Immunohistochemistry of BDNF+, p-CREB+, p-ERK1/2+, cells in the hippocampus. Con: control; MD: maternal deprivation; PD: PD98059. (×200)

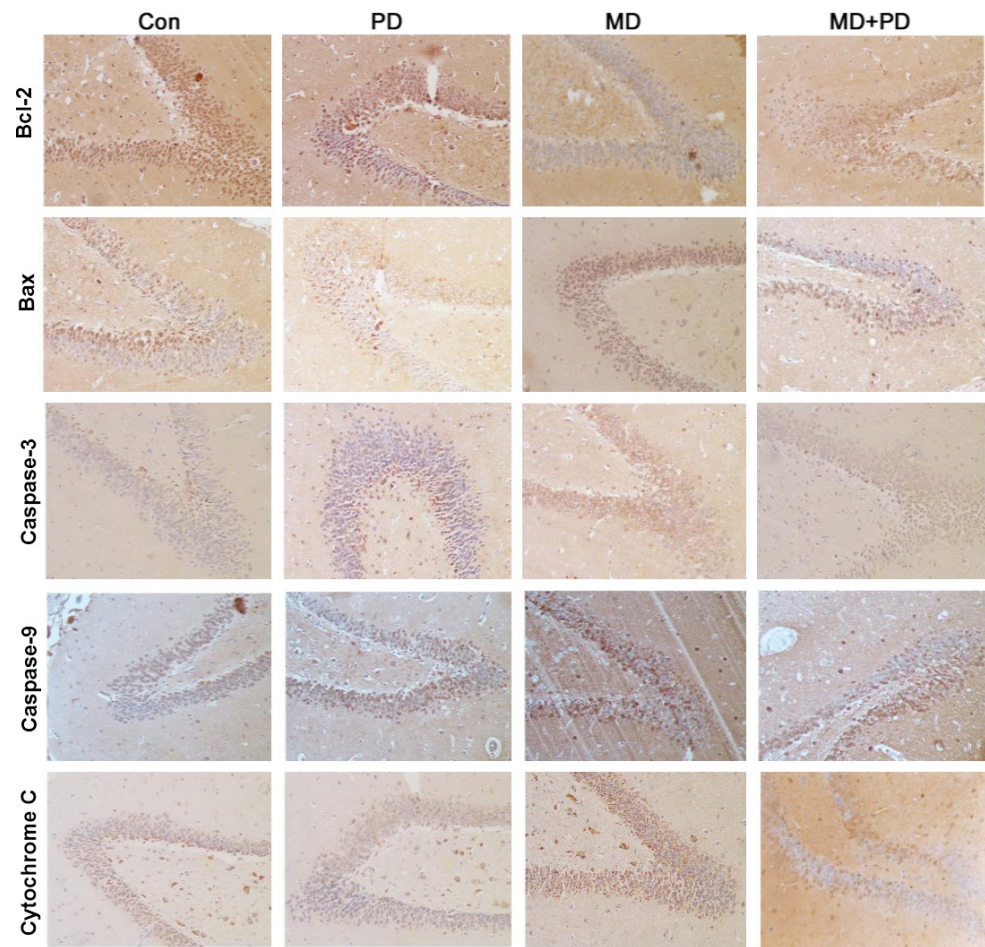


Figure 4. Immunohistochemistry of Bcl-2+, Bax+, caspase-3+, caspase-9+, and Cytochrome C+ cells in the hippocampus. Con: control; MD: maternal deprivation; PD: PD98059.(×200)

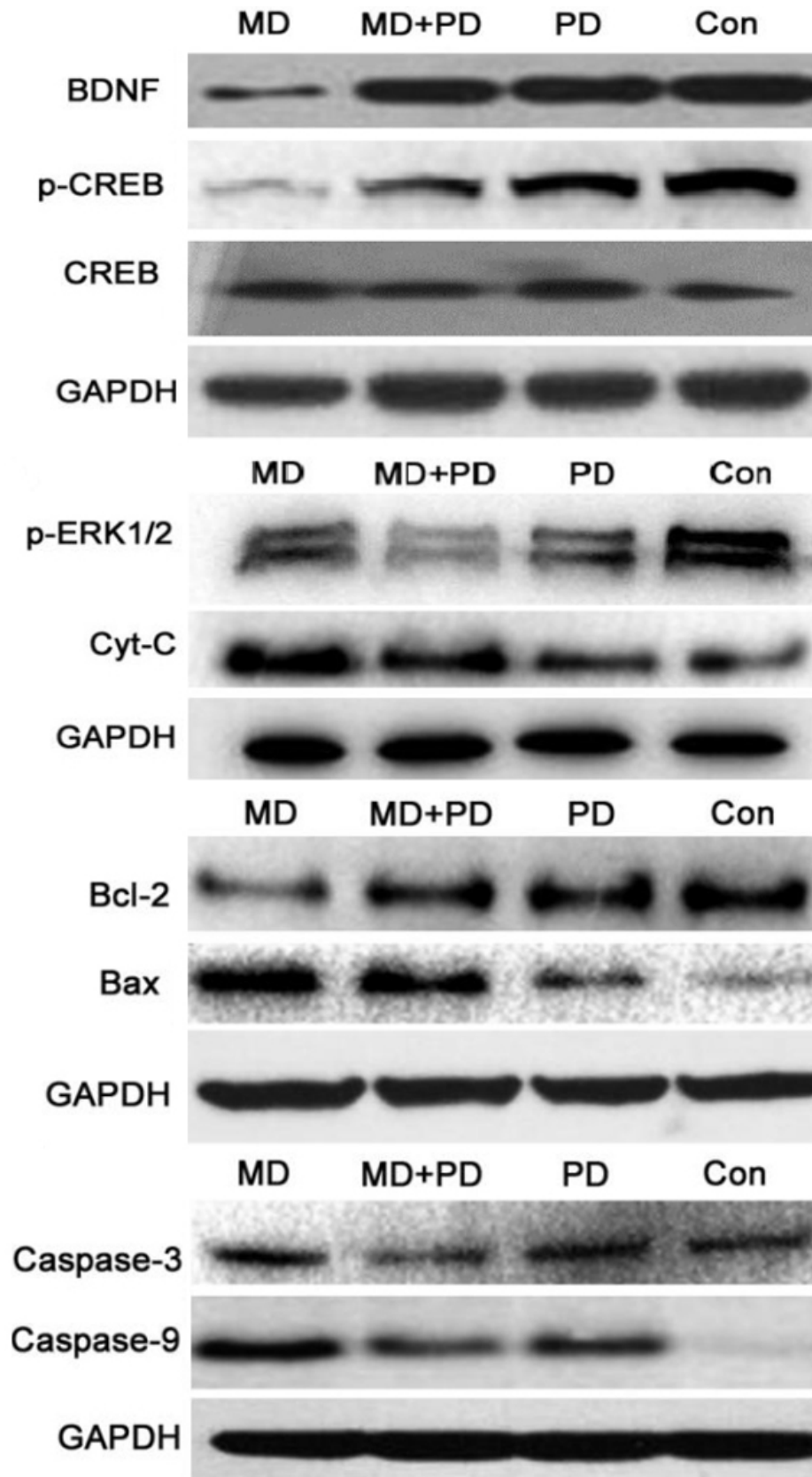


Figure 5. Protein expression levels of Bcl-2, BDNF, p-CREB, p-ERK1/2, Bax, caspase-3, caspase-9, and Cytochrome C in the hippocampus. Con: control; MD: maternal deprivation; PD: PD98059.

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Abbreviations: MD, Neonatal maternal deprivation; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; BDNF, brain-derived neurotrophic factor; CREB, cAMP responsive element-binding protein; p-CREB, phosphorylated CREB; p-ERK1/2, phosphorylation of ERK1/2; Bcl-2, B-cell lymphoma-2; PAGE, polyacrylamide gel electrophoresis

Key Words: Neonatal maternal Deprivation, Apoptosis, Signaling Pathway

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